AZELNIDIPINE ATTENUATES INFLAMMATORY RESPONSES, SUPEROXIDE AND RELEVANT SIGNALING PATHWAYS INDUCED BY AMYLOID-BETA IN MOUSE CEREBRAL ENDOTHELIAL CELLS

A Thesis

presented to

the Faculty of the Graduate School

at the University of Missouri-Columbia

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

TAO TENG

Dr. James C-M. Lee, Thesis Supervisor

DECEMBER 2014
The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

**AZELNIDIPINE ATTENUATES INFLAMMATORY RESPONSES, SUPEROXIDE AND RELEVANT SIGNALING PATHWAYS INDUCED BY AMYLOID-BETA IN MOUSE CEREBRAL ENDOTHELIAL CELLS**

Presented by Tao Teng,

A candidate for the degree of Master of Science,

And hereby certify that, in their opinion, it is worthy of acceptance.

________________________________________

Dr. James C-M. Lee, Department of Bioengineering

________________________________________

Dr. Shinghua Ding, Department of Bioengineering

________________________________________

Dr. Zezong Gu, Department of Path & Anat Sci-Anatomic Path
ACKNOWLEDGEMENTS

First I would like to express my sincere gratitude to my advisor Dr. James C-M. Lee for his support and guidance for my research. I am also grateful to Dr. Grace Y. Sun for allowing me to use her lab equipment during my research.

I would like to thank my committee member, Dr. Shinghua Ding and Dr. Zezong Gu, for their precious time and effort on my thesis and defense.

I appreciate all members in the laboratories of Drs. Jame C-M. Lee and Grace Y. Sun. for giving me invaluable help in my research. Specially, I would like to thank Andrey Tsoy, Brittani Bungart, Sholpan Askarova and Adrew Zorn for training me using lab facilities and teaching me useful techniques. Also, I appreciate Dr. Devin M. Ridgley for his great suggestion on thesis writing for me.

Finally, I would like to give special thanks to my family for their support. And my friends I have made here for making my life wonderful.
# TABLE OF CONTENTS

**ACKNOWLEDGEMENTS** ................................................................. ii  

**LIST OF ILLUSTRATIONS** ........................................................... v  

**Abstract** ................................................................................... vii  

**Chapter 1 Introduction** .............................................................. 1  
  1.1 Alzheimer’s disease ............................................................... 1  
  1.2 Amyloid-β peptide ............................................................... 2  
  1.3 Reactive Oxygen Species ..................................................... 4  
  1.4 ERK 1/2 and cPLA₂ .............................................................. 5  
  1.5 Nuclear factor kappa-light-chain-enhancer of activated B cells .. 6  
  1.6 Azelnidipine ...................................................................... 7  

**Chapter 2 Materials and Methods** ........................................... 9  
  2.1 Chemicals and Regents .......................................................... 9  
  2.2 Cell Culture ........................................................................ 11  
  2.2.1 Rat Primary Cortical Astrocytes .......................................... 11  
  2.2.2 bEnd.3 Mouse Cerebral Endothelial Cells ......................... 12  
  2.3 Preparation of Aβ1-42 .......................................................... 13
Chapter 3 Results ........................................................................ 20

3.1 Azelnidipine Suppresses Aβ-induced Superoxide Production in Both Primary Astrocytes and bEnd.3 Cells ............................... 20

3.2 Azelnidipine Suppresses Aβ-induced Phosphorylation of ERK 1/2 in bEnd.3 Cells ...................................................................... 26

3.3 Azelnidipine Suppressed Aβ-induced Phosphorylation of cPLA2 in bEnd.3 Cells ................................................................. 37

3.4 Azelnidipine Suppresses Aβ-induced translocation of NF-κB in bEnd.3 Cells .......................................................................... 43

Chapter 4 Discussion .................................................................... 46

Chapter 5 Conclusion and Future work ................................. 51

References ........................................................................ 52
LIST OF ILLUSTRATIONS

Figure 1.1 Chemical Structural of Azelnidipine ......................... 8

Figure 3.1 Effect of ALP on Aβ-induced ROS production in astrocytes ................................................................. 24

Figure 3.2 Effect of ALP on Aβ-induced ROS production in bEnd.3 mouse cerebral endothelial cells ............................. 25

Figure 3.3 Starving time study on ERK p42 phosphorylation in bEnd.3 cells ............................................................... 27

Figure 3.4 Starving time study on ERK p44 phosphorylation in bEnd.3 cells ............................................................... 28

Figure 3.5 Aβ treatment time course study on ERK p42 phosphorylation in bEnd.3 cells ................................................. 30

Figure 3.6 Aβ treatment time course study on ERK p44 phosphorylation in bEnd.3 cells ................................................. 31

Figure 3.7 Azelnidipine suppressed Aβ-induced phosphorylation of ERK p42 in bEnd.3 cells ............................................ 33

Figure 3.8 Azelnidipine suppressed Aβ-induced phosphorylation of ERK p44 in bEnd.3 cells ............................................ 35

Figure 3.9 Starving time-course study on cPLA₂ phosphorylation in bEnd.3 cells ......................................................... 39
Figure 3.10 Aβ treatment time course study on cPLA₂ phosphorylation in bEnd.3 cells ........................................... 40

Figure 3.11 Azelnidipine suppressed Aβ-induced phosphorylation of cPLA₂ in bEnd.3 cells ................................. 41

Figure 3.12 Effect of ALP on Aβ-induced NF-κB translocation in bEnd.3 cells .......................................................... 44

Figure 3.13 Representative immunofluorescent images of NF-κB p65 subunit and nucleus staining in bEnd.3 cells ......... 45

Figure 4.1 Schematic of relative pathways ........................................... 47
Abstract

Alzheimer’s disease (AD) is a debilitating neurodegenerative disease with no known cure. The cause and progression of AD is still unclear. Over the years, studies have shown that one of the earliest cytotoxic effects is the production of reactive oxygen species (ROS) and inflammation induced by the amyloid-β peptide in microglial cells, endothelial cells and neurons. The focus of this thesis is to study the effects of Azelnidipine (ALP), which is a Calcium channel blocker, on Aβ-induced oxidative stress and its downstream pathways in mouse immortalized cerebral endothelial cells (bEnd.3). In AD, Aβ_{1-42} induces oxidative stress through the activation of ERK 1/2 pathway, phosphorylation of cPLA₂ and production of intercellular superoxide anions. Furthermore, Aβ_{42} has been shown to induce the translocation of NF-κB into the nucleus of bEnd.3 cells causing an inflammatory response. Here, ALP is utilized to reduce the ROS and inflammatory effects of Aβ_{42} in bEnd.3 cells. The results show that ALP is effective in reducing the Aβ_{42} induced superoxide anion production, ERK 1/2 activation, cPLA₂ phosphorylation and NF-κB translocation into the nucleus of bEnd.3 cells. Thus, ALP may be an effective treatment to alleviate the debilitating effects of AD. Finally, this research implicates that there may be cross-talk between the ERK 1/2 pathway and NF-κB translocation into the bEnd.3 nucleus.
Chapter 1 Introduction

1.1 Alzheimer’s disease

Alzheimer’s disease (AD) is a debilitating neurodegenerative disease first discovered by German physician and neuropathologist Alois Alzheimer in 1906 (Berchtold & Cotman, 1998). AD is the most common form of dementia and is characterized by the spontaneous misfolding of proteins, specifically Amyloid-β (Aβ), into amyloid plaques (micrometer scale) and oligomers (nanometer scale) (Chiti & Dobson, 2006, Jan et al., 2010, Karran et al., 2011, Selkoe, 2000b). In the case of AD, amyloid structures form in the brain and inhibit cellular function by blocking neuron synapsis, which eventually results in death (Kim et al., 2013, Lacor et al., 2007, Lauren et al., 2009). There is no known drugs targeting Aβ showed positive results in clinical trials yet (Morris, 2013). AD is a widespread disease that affects approximately 5.2 million Americans annually, with the vast majority being age 65 or older, according to the latest estimates in 2014 (Hebert et al., 2013) (Association, 2006; 2014). Over the last few decades modern medicine has increased the average life expectancy and as a result, AD is one of the few diseases that has increased in occurrences and fatalities over the same time span (Association, 2006) (Minino et al., 2002, Murphy et al., 2013). Worldwide, in 2006 there were 26.6 million people with AD
which is expected to increase to affect 1 in 85 people by 2050 (Brookmeyer et al., 2007).

1.2 Amyloid-β peptide

Although the pathogenesis is still unclear, people believe that the Amyloid-β peptide (Aβ) is responsible for AD progression. One hypothesis is that the dementia like symptoms of AD arise from the deposition and aggregation of Aβ into plaques (Selkoe, 2000a, Hardy & Selkoe, 2002) (Stalder et al., 1999, Dickson, 1999, Frautschy et al., 1998, Selkoe, 2000a).

Aβ is derived from the amyloidogenic pathway processing of amyloid precursor protein (APP) which involves β-secretase (Vassar, 2004). APP is an integral membrane protein concentrated in the synapse of neurons but expressed in many tissues, it is reported to have a role in cell adhesion (Sosa et al., 2013). APP is cleaved and processed by secretase enzymes into two pathways, the non-amyloidogenic and amyloidogenic pathway. Under the non-amyloidogenic pathway, which is the normal cleavage pathway, APP is first cleaved by α-secretase into soluble APPα (sAPPα) and the carboxy-terminal fragment C83 at a site inside the Aβ sequence domain. C83 is then cleaved by γ-secretase into P3 and the APP intracellular domain (AICD C57/59) (Thathiah & De Strooper, 2011).
Under the amyloidogenic pathway, also known as the abnormal cleavage pathway, APP is cleaved by β-secretase forming sAPPβ and the carboxy-terminal fragment C99, followed by γ-secretase cleavage and produce AICD (C57/59), Aβ_{40}, Aβ_{42} or other Aβ isoforms (King & Scott Turner, 2004, Koo, 2002, Reinhard et al., 2005). sAPPα has been found to have neuroprotective and neuritotrophic properties (Chen & Yankner, 1991, Jin et al., 1994, Mattson et al., 1993, Schubert et al., 1989, Furukawa et al., 1996a, Han et al., 2005, Ma et al., 2009). It is also shown that sAPPα regulates the proliferation of embryonic and adult neural stem cells as a growth factor (Caille et al., 2004, Ohsawa et al., 1999). In vivo studies indicate that sAPPα alone has the ability to eliminate the abnormalities of APP deficient mice (Ring et al., 2007). Unlike sAPPα, sAPPβ does not have neuroprotective effects (Furukawa et al., 1996a, Furukawa et al., 1996b). The AICDs are quickly degrade after γ-cleavage, but it forms a stable complex with Fe65 and Tip60, this complex promotes the gene expression of such as KAI1, Neprilysin, LRP1, p53, GSK-3β and EGF receptor (Alves da Costa et al., 2006, Baek et al., 2002, Cao & Sudhof, 2004, Kim et al., 2003, Liu et al., 2007, Pardossi-Piquard et al., 2005, Zhang et al., 2007).

Among all Aβ peptides, Aβ_{40} is abundantly produced in both healthy and AD patients’ brains, whereas other forms of Aβ are produced at lower levels, including Aβ_{37}, Aβ_{38}, Aβ_{42} and Aβ_{43} (Benilova et al., 2012). Aβ_{42}
is suggested to be the most toxic form, and the ratio of $A\beta_{42}/A\beta_{40}$ is considered to be a biomarker for AD diagnosis (Klein et al., 1999, Kuperstein et al., 2010, Spies et al., 2010, Wiltfang et al., 2007). Both $A\beta_{40}$ and $A\beta_{42}$ peptides attempt to self-aggregate, but $A\beta_{42}$ is more likely to aggregating from its monomer form into higher molecular weight forms, such as $A\beta$-derived diffusible ligand (ADDL), low-molecular weight oligomers, oligomers, protofilaments, fibrils and plaques (Cleary et al., 2005, Jan et al., 2010, Mucke et al., 2000, Resende et al., 2008, Walsh et al., 2002, Westerman et al., 2002). Different species of $A\beta_{42}$ show different biological effects, studies show that the oligomer form of $A\beta$ is believed to be more neurotoxic than the monomer or fibril form (Resende et al., 2008, Walsh & Selkoe, 2007), whereas $A\beta$ dimmers and trimmers are toxic to synapses (Klyubin et al., 2008, Walsh et al., 2005, Benilova et al., 2012).

### 1.3 Reactive Oxygen Species

Cells in the central nervous system (CNS) are highly sensitive to oxidative stress due to the high oxygen consumption within the brain. Oxidative stress is reported to be involved in a number of neurodegenerative disease, including AD (Coyle & Puttfarcken, 1993, Emerit et al., 2004). Numerous studies have demonstrated that
oxidative stress in cerebral vasculature and astrocytes is induced by vascular deposition of Aβ (Abramov & Duchen, 2005, Cai et al., 2003). Reactive oxygen species (ROS) play an important role in the modulation of cellular metabolic pathways under normal conditions (Suzuki et al., 1997). When over produced, which is associated with Aβ-induced oxidative stress (Girouard & Iadecola, 2006, Park et al., 2008, Sim et al., 2005), ROS becomes harmful and causes Aβ-induced cytotoxic effects (Butterfield et al., 2001, Sultana & Butterfield, 2010).

1.4 ERK 1/2 and cPLA₂

Aβ has been shown to induce cytotoxic effects that alter the activation of extracellular signal-regulated kinase 1/2 (ERK 1/2) which are also known as mitogen-activated protein kinase 3 (MAPK3), MAPK 1 cascade. Also, Aβ was reported to induce the phosphorylation of cytosolic phospholipase A₂ (cPLA₂) (Dineley et al., 2001, McDonald et al., 1998, Moses et al., 2006, Shelat et al., 2008, Stephenson et al., 1996, Young et al., 2009, Zhu et al., 2006). ERKs are widely expressed protein kinase intracellular signaling molecules. The ERK pathway can be activated by many different stimuli, such as growth factors, cytokines, virus infection etc. ERKs can activate many transcription factors (Rao & Reddy, 1994) and some downstream protein kinases, including cPLA₂.
phosphorylation (Askarova et al., 2011b, Lee et al., 2011, Zhu et al., 2009).

Phospholipases A2 (PLA2s) are enzymes that catalytically hydrolyze the sn-2 acyl bond of phospholipids and release arachidonic acid (Murakami & Kudo, 2002, Sun et al., 2004), which is converted into active compounds called eicosanoids by downstream modification of cyclooxygenases. Eicosanoids are signaling molecules classified as anti-inflammatory and inflammatory mediators (Dennis, 1994). PLA2s enzymes currently consists of 15 groups and many subgroups, but mainly categorized into three major families: secreted PLA2s (sPLA2), the cytosolic PLA2s (cPLA2) and calcium-independent PLA2s (iPLA2) (Schaloske & Dennis, 2006). It is reported that overproduction of cPLA2 is involved in many neurodegenerative diseases, including AD (Stephenson et al., 1996, Sun et al., 2007).

1.5 Nuclear factor kappa-light-chain-enhancer of activated B cells

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a protein complex that controls transcription of DNA. NF-κB is found to be involved in cellular responses stimulated by stress, cytokines, free radicals, ultraviolet irradiation, oxidized LDL and
bacterial or viral antigens (Tian & Brasier, 2003, Perkins, 2007, Gilmore, 2006, Brasier, 2006, Gilmore, 1999). NF-κB has also been reported to be involved in process of synaptic plasticity and memory (Levenson et al., 2004, Albensi & Mattson, 2000, Merlo et al., 2002, Freudenthal et al., 1998, Meffert et al., 2003). NF-κB complex contains two subunits, class I protein like p50 and p52 and class II protein like RelA (p65), RelB and c-Rel (Nabel & Verma, 1993). In unstimulated cells, NF-κB dimers are sequestered in the cytoplasm by its inhibitor, IκBs (Inhibitor of κB), it covers the nuclear localization signals (NLS) of NF-κB to keep it in an inactive state in the cytoplasm (Jacobs & Harrison, 1998). Upon stimuli, the IκB proteins are degraded by IκB kinase (IKK), then NF-κB complex is free and translocates to the nucleus for gene expression.

1.6 Azelnidipine

Azelnidipine (ALP) (Fig. 1.1) is a dihydropyridine calcium channel blocker that has a long-lasting hypotensive effect (Oizumi et al., 1989). Studies suggest that dihydropyridine (DHP) compounds such as ALP facilitate Aβ_1-42 clearance across the blood-brain barrier (BBB) (Bachmeier et al., 2011). Also, Azelnidipine is reported to have anti-inflammatory and neuroprotective effects (Kurobe et al., 2013, Omote et al., 2014). Evidence suggests that ALP may inhibit inflammatory
responses triggered by different stimuli, such as TNF-α, through its anti-oxidative properties (Matsui et al., 2005, Ohyama et al., 2012, Yamagishi et al., 2004). Here we investigate the effects of ALP on the aforementioned proteins and processes shown to be associated with Aβ-induced cytotoxic effects in AD progression (ROS production and inflammation). The results indicate that ALP reduces the inflammation and ROS effects of Aβ buildup within the human brain. Thus, ALP may be a treatment option to alleviate the debilitating effects of AD.

Figure 1.1 Chemical Structural of Azelnidipine
Chapter 2 Materials and Methods

2.1 Chemicals and Regents

Dulbecco’s Modified Eagle Medium with phenol red and high glucose (1X) (DMEM) (A14431), Rat Primary Cortical Astrocytes (N7745-100), 0.25% or 0.05% Trypsin-EDTA (1X) (25200-056), Dulbecco’s Phosphate Buffered Saline without Calcium Chloride or Magnesium Chloride (1X) (DPBS) (14190-144), Penicillin-Streptomycin (5,000 Units/ml) (P/S) (15070-063), Dulbecco’s Modified Eagle Medium without phenol red (1X) (DMEM w/o phenol red) (31053-028) and UltraPure™ 10% Sodium Dodecyl Sulfate Solution (10% SDS) (15553-035) are from Life Technologies (Carlsbad, CA, USA). Fetal Bovine Serum (FBS) (F6178-500ml), Dimethyl Sulfoxide Hybri-max (DMSO) (D2650), Dihydroethidium (DHE) (37291-25MG), Azelnidipine (ALP) (A7106-10MG), 1, 1, 1, 3, 3, 3-Hexafluoro-2-propanol (HFIP) (Fluka) (52517), Triton® X-100 (X100-100ML), Paraformaldehyde (powder, 95%) (PFA) (158127-500G), Hoechst 33258 (861405-100MG), Agarose (A9539-50G) and β-actin antibody are from Sigma-Aldrich (St. Louis, MO, USA). bEnd.3 Mouse Cerebral Endothelial Cell Line (bEnd.3) (ATCC CRL-2299) is from American Type Culture Collection (ATCC, Manassas, VA, USA). Bovine Serum Albumin (Fraction V) (BP1605-100), Glycerol (G33-1) and Methanol (A413-4) are from Thermo Fisher Scientific (Hampton, NH,
USA). Beta-Amyloid (1-42) (Human) (20276) is from Anaspec (Fremont, CA, USA). Ham’s F12 (without phenol red) (226-057-12) is from Crystalgen (Long Island, NY, USA). NF-κB p65 (c-20) Antibody (SC-372) is from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-Rabbit IgG Fab2 Alexa Fluor 488 Molecular Probes (4412s), Phosphate Buffered Saline (PBS-20X) (9808), 3X Blue Loading Buffer (3X Blue Loading Buffer & 30X Dithiothreitol (DTT)) (7722s), Protease/Phosphatase Inhibitor Cocktail (100X) (5872s), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (197G2), Rabbit Monoclonal Antibody (Phos-ERK1/2) (4377), p44/42 MAPK (Erk1/2) (3A7), Mouse Monoclonal Antibody (ERK1/2) (9107), Phospho-cPLA2 (Ser505) Antibody (cPLA2) (2831), cPLA2 Antibody (cPLA2) (2832), Anti-Rabbit IgG, HRP-linked Antibody (7074s) and Anti-Mouse IgG, HRP-linked Antibody (7076P2) are from Cell Signaling Technology (CST, Danvers, MA, USA). Recombinant Mouse (E. coli-derived) Tumor Necrosis Factor-α (TNF-α) (aa84-235) (410-TRNC) is from R&D systems™ (Minneapolis, MN, USA). Resolving Gel Buffer (1.5M Tris-HCl buffer, pH 8.8) (161-0798), Stacking Gel Buffer (0.5M Tris-HCl buffer, pH 6.8) (161-0799), 30% Acrylamide/Bis Solution (37.5:1) (161-0158), 10X Tris Buffered Saline (170-6435), Tween 20 (170-6531), Nitrocellulose Membrane (0.45 μm) (162-0115), 10X Tris/Glycine/SDS Buffer (TGS buffer) (161-0772) and 10X Tris/Glycine Buffer (TG buffer) (61-0771) are from Bio-RAD Laboratories.
2.2 Cell Culture

2.2.1 Rat Primary Cortical Astrocytes

Rat primary cortical Astrocytes were seeded into T25 cell culture flask (Corning, NY, USA) with cell culture medium (15% FBS (v/v), 1% P/S (v/v), DMEM) at a ratio of 1:9, totally 10ml diluted cell solution was seeded into each T25 cell culture flask. The cells were maintained at 37°C in a 5% CO2 humidified incubator, cell culture medium was changed every other day and cells were sub-cultured into another T25 cell culture flask before reaching confluence. Briefly, cells were washed with pre-warmed PBS for twice, 2 ml 0.05% Trypsin/EDTA solution was added into each T25 cell culture flask and incubated in the incubator for 2-5 minutes, cells were then observed under a microscope to see whether detached from the substrate of the flask. After detaching, 8ml
cell culture medium was added into the flask to stop the reaction. Cell solution was then diluted with cell culture medium with a ratio of 2:8 and then added into a new T25 cell culture flask and maintained in the incubator. For experiment, cells were sub-cultured into dishes with the same dilution ratio. Only three passages of Astrocytes were used for experiments.

2.2.2 bEnd.3 Mouse Cerebral Endothelial Cells

Passage 21 bEnd.3 mouse cerebral endothelial cell line was purchased from ATCC. Cells were seeded into T25 cell culture flask after thawing in a 37°C water bath. 9 ml cell culture medium (10% FBS (v/v), 1% P/S (v/v), DMEM) was then added into the flask. Cell culture medium was changed every two to three days. After confluence, cells were washed twice with pre-warmed DPBS, 2ml 0.25% Trypsin/EDTA solution was added into the flask, and the flask was incubated for 2-3 minutes until cells were fully detached. 8ml freeze medium (95% cell culture medium, 5% DMSO (v/v)) was added into the flask after cells detached. 1ml cell-freeze medium solution was then added into each “O” ring cryopreservation vials. The vials were put into a Nalgene® Mr. Frosty™ Cryo 1°C freezing container (Rochester, NY, USA) (5100-001) which was then placed in the -80°C refrigerator for at least 4 hours to achieve
a -1°C/min. rate of cooling. After cooling down, cryopreservation vials were stored in a liquid nitrogen tank for long-term storage.

Passage 22 (P.22) bEnd.3 cell line were used for experiments. Cells were seeded into T25 cell culture flasks with cell culture medium (10% FBS, 1% P/S, DMEM). The flasks were maintained at 37°C in a 5% CO2 humidified incubator, cell culture medium was changed every two to three days. Before confluence, cells were sub-cultured into a new T25 cell culture flask. For experiments, cells were also sub-cultured into dishes, 6-well or 12-well plates.

2.3 Preparation of Aβ1-42.

1 mg powdered Aβ1-42 peptide was dissolved in 220µl of HFIP, solution was milk-like now. The vial containing dissolved Aβ1-42 was vortexed for 5 minutes, wrapped with Aluminum foil and placed under a hood for 1 hour. The Aβ1-42-HFIP solution became transparent after 1 hour. Afterwards, 10µl of Aβ1-42 was aliquoted into 20 Eppendorf tubes. Then, HFIP in each tube was removed by placing tubes in a speed vacuum apparatus for exactly 1 hour. After 1 hour, tubes were removed from the speed vacuum, sealed with Parafilm® and stored at -20°C until use. Normally, Aβ1-42 was freshly made every month.
When used, the Aβ1-42 film was resuspended in 2μl DMSO and sonicated for 5 minutes with a VWR® sonicator (Radnor, PA, USA). 98 μl DPBS was then added into the tube and vortexed for 60 seconds. The 100 μM concentration of the Aβ1-42 solution was now 100μM. Tube was then incubated at 4°C for 45-48 hours to allow oligomerization. The solution was then further diluted in treating medium (1% BSA (w/v), DMEM) to a final concentration of 1μM or 5μM for treatments.

2.4 Preparation of Azelnidipine

Azelnidipine was aliquoted into Eppendorf tubes and scaled, the weight of Azelnidipine in each tube was recorded for further calculation. Azelnidipine was first dissolved in 1 ml DMSO (solution A). Then, 10μl solution A was added into 990 μl treating medium (1% BSA, DMEM) (Solution B). Solution B was added to 10 ml or 20 ml of treating medium to make a final concentration of 20 nM (Final solution). This final solution was used for cell pre-treatment.

2.5 Reactive Oxygen Species (ROS) Measurement

To quantify superoxide anion production induced by Aβ1-42 and suppressed by Azelnidipine in astrocytes or bEnd.3 mouse cerebral
endothelial cells, cells were starved for 12 hours in DMEM, followed by pre-treatment with 20 nM Azelnidipine for 4 hours. After pre-treatment, cells were treated with 5 μM Aβ1-42 and 20 μM DHE for 2 hours. DHE was first dissolved in DMSO and further diluted in phenol red DMEM to achieve the desired final concentration.

Fluorescent intensity of DHE was measured at room temperature using a Nikon TE-2000U fluorescence microscope (Tokyo, Japan) with a 20X objective lens. For each dish, 20 bright field images and 20 DHE fluorescence images were acquired using a CCD camera from Photometrics (Turson, AZ) controlled by a PC running MetaVue imaging software v6.2r6 (Universal Imaging, PA). Fluorescent intensity of DHE per cell was measured by dividing image fluorescent intensity by cell number of the image. Prior to data analysis, the background was subtracted from all images and the threshold was used for each image as further background subtraction. The threshold level was maintained for all images within the same experiment.

2.6 NF-κB Translocation Measurement

For quantification of translocation of NF-κB, immunofluorescent technology was employed. Cells were sub-cultured into 12-well plates containing pre-coated coverslip each well. After reaching 70-80%
confluence, cells were starved for 12 hours and pre-treated with 20 nM Azelnidipine for 4 hours. Cells were then treated with 5 μM Aβ1-42 or 100 ng/ml TNF-α for 2 hours, fixed with 3.7% PFA (w/v) for 15 minutes with rotation, permeabilized by incubating with 0.1% Triton® X-100 (v/v) for 8 minutes, blocked with 5% BSA (w/v) in DPBS for 1 hour, and then incubated with NF-κB p65 (C-20) rabbit polyclonal IgG primary antibody (1:50 dilution, diluted in 1% BSA in DPBS) overnight for NF-κB staining. After approximately 16 hours, cells were incubated with anti-rabbit IgG (H+L), F(ab’)2 fragment (Alexa Fluor® 488 conjugate) secondary antibody (1:1000 dilution, diluted in 1% BSA in DPBS) for 1 hour. The cell nuclei were then stained with Hoechst 33258 (2 μg/ml, diluted in DPBS) for 10 minutes. Between each step, cells were washed with DPBS for 3-5 minutes. After staining, coverslips were allowed to stay in a dark room for approximately 20 minutes to dry. Then each coverslip was mounted onto a glass slide and sealed with nail polish. Images were acquired within two days.

Fluorescent images of NF-κB and nucleus were acquired using the same Nikon microscope as earlier with a 60X objective. Twenty images of NF-κB and nucleus were taken, respectively. Background subtraction was done before image acquirement. To quantify NF-κB translocation, total image intensity (ITotal) and nuclear intensity (INucleus) of NF-κB p65 were measured for each fluorescent image. Nuclear intensity
divided by total intensity (INucleus/ITotal) was used to represent NF-κB translocation.

2.7 Western Blot Analysis

Western blot analysis was employed to measure ERK 1/2 or cPLA₂ phosphorylation. Cells were sub-cultured onto a 6-well plates and the experiment was carried out when cells reached 90% confluence. For treatments, cells were starved for 24 hours before being pre-treated with 20 nM Azelnidipine for 4 hours, followed by 5 µM Aβ1-42 treatment for 15 minutes or 30 minutes for measurement of phosphorylation of ERK 1/2 or cPLA₂, respectively. After treatments, cells were washed with pre-warmed DPBS twice and lysed by blue loading buffer (62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% (w/v) SDS, 10% glycerol, 0.01% (w/v) bromophenol blue, 41.7 mM Dithiothreitol, Aprotinin, Bestatin, E64, Leupeptin, β-glycerophosphate, sodium pyrophosphate and sodium orthovanadate). Cells were collected from the 6-well plates, put into Eppendorf tubes and immediately placed on ice to prevent protein degradation. Samples were then sonicated for 10 seconds three times, micro centrifuged for 10 seconds and boiled for 5 minutes at 95°C. After cooling down, samples were stored at -20°C for further experimentation.
After thawing, equivalent amounts of 40 μl samples were subjected to electrophoresis in 10% SDS-polyacrylamide gels, and transferred to 0.45 μM nitrocellulose membranes. Membranes were blocked in 5% (w/v) non-fat milk in Tris-buffer saline containing 0.1% (v/v) Tween 20 (TBST) for 1 hour and then incubated overnight at 4°C with primary antibodies in 5% (w/v) BSA or 5% (w/v) non-fat milk in TBST with dilution ratio in accordance with the antibody datasheet, p-cPLA₂ or cPLA₂ antibodies (1:1000 dilution), p-ERK1/2 (1:1000 dilution) or ERK 1/2 (1:2000 dilution) antibodies and β-actin antibody (1:50,000 dilution). Membranes were washed 5 minutes three times and incubated with horseradish peroxidase conjugated anti-rabbit IgG (H&L), antibody (1:3000 dilution) or horseradish peroxidase conjugated anti-mouse IgG (H&L) antibody (1:5000 dilution) in 5% (w/v) non-fat milk in TBST at room temperature for 1 hour. After washing for 5 minutes three times with TBST, the membrane was subjected to Pico or Femto Chemiluminescent detection reagents to visualize bands. Bands were detected by blue autoradiography film or Fuji LAS-3000 image reader (GE Healthcare, Piscataway, NJ, USA). Bands intensity were quantified using a computer running Quantity One software v4.6.6 (Bio-Rad, Hercules, CA, USA). For re-incubation with antibodies, membranes were stripped with Restore™ Western Blot Stripping Buffer for 20 minutes and washed for 5 minutes (x6) with TBST. After washing,
membranes were good for re-incubation with antibodies, each membrane was stripped no more than twice.

2.8 Statistical Analysis

The data is presented as the mean ± standard deviation (SD) from at least three trials of a given experiment. Analysis was carried out with student-t test utilizing KaleidaGraph. Values of $p < 0.05$ are considered statistically significant.
Chapter 3 Results

3.1 Azelnidipine Suppresses Aβ-induced Superoxide Production in Both Primary Astrocytes and bEnd.3 Cells

In order to investigate the effects of ALP on Aβ-induced superoxide production and determine the working concentration of ALP, intercellular ROS’s of rat primary cortical astrocytes and mouse cerebral endothelial cells (bEnd.3 cell line) were fluorescently labeled with DHE. Astrocytes and bEnd.3 cells were exposed to the oligomer form of Aβ1-42 for 2 hours as a control which showed an increase in superoxide anions with respect to the cells pre-treated with 20 nM ALP or cells without treatment (Figures 3.1 and 3.2) (Figure 3.2 contributed by Andrey Tsoy). Figures 3.1a and 3.1b show the effect of ALP on the astrocytes is concentration dependent. A 20 nM pre-treatment of ALP proved to be the most effective at preventing the superoxide production induced by Aβ1-42 with lower ALP concentrations (10 nM) being ineffective and higher ALP concentrations (100 nM) inducing its own ROS production. When pre-treated with 20 nM ALP the Aβ1-42 induced superoxide production was reduced 25% and 52% for astrocytes and endothelial cells, respectively, when compared to the 5 µM Aβ1-42 positive control sample. (Figures 3.1a and 3.2) Therefore, 20 nM ALP was used for the subsequent experiments.
c.

Bright Field

Control

5 μM Aβ

20 nM ALP

10 nM ALP

5 μM Aβ + 20 nM ALP

5 μM Aβ + 10 nM ALP

DHE
Figure 3.1 Effect of ALP on Aβ-induced ROS production in astrocytes

a) Astrocytes were treated with two concentrations of ALP (10 and 20 nM), 5 μM Aβ₁₋₄₂, both ALP and Aβ₁₋₄₂ or neither compound (Control) to yield a total of six data sets. b) Astrocytes were pre-treated with 200 nM ALP, treated with 5 μM Aβ₁₋₄₂, both ALP and Aβ₁₋₄₂ or neither compound (Control) to yield a total of 4 data sets. c) Representative fluorescent images of DHE ROS intensity. ROS production was quantified by measuring the DHE intensity within each sample. The data is expressed as a fraction percentages of the control group with the mean ± SD from three independent experiments reported (** p<0.01, *** p<0.001, comparing with control group) (o p<0.05, oo p<0.01, comparing with 5 μM Aβ₁₋₄₂ treatment only group).
bEnd.3 cells were treated with 20 nM ALP, 5 μM Aβ₁₋₄₂, both ALP and Aβ₁₋₄₂ or neither compound (Control) to yield a total of 4 data sets. ROS production was quantified by measuring the DHE intensity within each sample. The data is expressed as a fraction percentages of the control group with the mean ± SD from three independent experiments reported (**: p<0.01, ***: p<0.001, comparing with control group) (**: p<0.01, comparing with 5 μM Aβ₁₋₄₂ treatment only group) (Data contributed by Andrey Tsoy).
3.2 Azelnidipine Suppresses Aβ-induced Phosphorylation of ERK 1/2 in bEnd.3 Cells

In an effort to determine the most effective method for studying how Aβ1-42 contributes to ERK p42 and p44 phosphorylation, bEnd.3 cells were starved over a 24 hour period and phosphorylated ERK (p-ERK) p42/p44 levels were measured periodically. The data for both ERK p42 and ERK p44 showed an initial increase in activation (5 minutes) and an immediate suppression thereafter to reach a minimum at 30 minutes (Figure 3.5 & Figure 3.6). Both systems continued to suppress ERK 1/2 phosphorylation up to 24 hours. A 24 hour starvation time was used for subsequent experiments due to the low expression of p-ERK p42 and p44.
Figure 3.3 Starving time study on ERK p42 phosphorylation in bEnd.3 cells

bEnd.3 cells were starved over a 24 hour period and p-ERK p42 level was measured periodically. Protein expression level was quantified by western blot analysis. The data is expressed as a fraction percentages of the control group with box plot from three independent experiments reported. (* p<0.05, ** p<0.01, *** p<0.001, comparing with non-starving group).
Figure 3.4 Starving time study on ERK p44 phosphorylation in bEnd.3 cells

bEnd.3 cells were starved over a 24 hour period and p-ERK p44 level was measured periodically. Protein expression level was quantified by western blot analysis. The data is expressed as a fraction percentages of the control group with box plot from three independent experiments reported. (* p<0.05, *** p<0.001, comparing with non-starving group).
To assess the effects of Aβ1-42 on ERK 1/2 phosphorylation a similar time-course study was carried out. bEnd.3 cells were treated with Aβ1-42 for a period of 22 hours where p-ERK levels were measured periodically. Phosphorylation ratio of ERK p42 was quantified by dividing p-ERK p42 intensity by total ERK p42 intensity (shown as normalized by total) or subsequently divided by β-actin intensity (shown as normalized by β-actin) (Figure 3.7 & Figure 3.8). β-actin served as loading control, which shows that the loading procedure of western blot analysis had a minimum influence on quantitative results (Figure 3.7 & Figure 3.8). The results revealed that bEnd.3 cells treated with Aβ1-42 produced more p-ERK p42 compared to the control group cells which did not receive any Aβ1-42 treatment with a maximum increase of 1.7-fold at 15 minutes (Figure 3.7). Aβ1-42 treatment induced similar phosphorylation of ERK p44 where p-ERK p44 reached a maximum at 15 minutes which was 2 times greater than the previous time point (Figure 3.8). Future bEnd.3 cells were treated with Aβ1-42 for 15-minutes to induce the maximum p-ERK p42/p44 expression.
Figure 3.5 Aβ treatment time course study on ERK p42 phosphorylation in bEnd.3 cells

bEnd.3 cells were treated with 5 μM Aβ1-42 over a 22 hour period, p-ERK p42 level was measured periodically. The phosphorylation ratio of ERK p42 was quantified by dividing p-ERK p42 intensity by total ERK p42 intensity (shown as normalized by total) or subsequently divided by β-actin intensity (shown as normalized by β-actin). Protein expression level was measured by western blot analysis. The data is expressed as true values from a single trial.
bEnd.3 cells were treated with 5 μM Aβ_{1-42} over a 22 hour period, p-ERK p44 level was measured periodically. Protein expression level was quantified by western blot analysis. The data is expressed as true values from a single trial.
To investigate effects of ALP on Aβ-induced ERK 1/2 phosphorylation, four bEnd.3 cell treatment groups were used; a control group without treatment, Aβ group with 5 µM Aβ_{1-42} treatment for 15 minutes, ALP + Aβ_{1-42} group with pretreatment of 20 nM ALP for 4 hours and treatment of 5 µM Aβ_{1-42} for 15 minutes and ALP group with 20 nM ALP pretreatment for 4 hours. A 1.3-fold increase of p-ERK p42 expression was observed for the Aβ_{1-42} group but not the groups with ALP treatment when compared to the control group (Figure 3.9). Similarly, a 2.4-fold increase was observed for p-ERK p44 expression within the Aβ_{1-42} group but not the groups with ALP treatment when compared to the control group (Figure 3.10). The results clearly indicate that ALP is capable of suppressing the effects of Aβ_{1-42} induced phosphorylation of ERK 1/2 with little to no additional phosphorylation from ALP as shown with the 20 nM ALP treatment groups.
Figure 3.7 Azelnidipine suppressed Aβ-induced phosphorylation of ERK p42 in bEnd.3 cells

bEnd.3 cells were treated with 20 nM ALP for 4 hours, 5 μM Aβ1-42 for 15 minutes, both ALP and Aβ1-42 or neither compound (Control) to yield a total of four data sets. The phosphorylation level of ERK p42 was quantified by measuring the protein expression levels of both ERK p42 and p-
ERK p42. The data is expressed as a fraction percentages of control group with the mean ± SD from four independent experiments reported (* p<0.05, *** p<0.001, comparing with control group) (oo p<0.01, ooo p<0.001, comparing with 5 μM Aβ1-42 treatment only group).
Figure 3.8 Azelnidipine suppressed Aβ-induced phosphorylation of ERK p44 in bEnd.3 cells

bEnd.3 cells were treated with 20 nM ALP for 4 hours, 5 μM Aβ1-42 for 15 minutes, both ALP and Aβ1-42 or neither compound (Control) to yield a total of four data sets. The phosphorylation level of ERK p44 was quantified by measuring the protein expression levels of both ERK p42 and p-ERK p42. The data is expressed as a fraction percentages of control group with the mean ± SD.
from four independent experiments reported (** p<0.01, comparing with control group) (oo 
p<0.01, ooo p<0.001, comparing with 5 μM Aβ1-42 treatment only group).
3.3 Azelnidipine Suppressed Aβ-induced Phosphorylation of cPLA₂ in bEnd.3 Cells

Just as in ERK 1/2, we did a starving time-course study for cPLA₂ phosphorylation. Unlike ERK 1/2, the ratio of p-cPLA₂/cPLA₂ showed little change with respect to starving time (Figure 3.11). Western blot analysis showed that with 30 minutes starving time, p-cPLA₂ expression was lowest in bEnd.3 cells, but the decrease was only ~20% compared to the control group. All other starving times show no significant difference between starving groups and control group. The cells were starved for 24 hours in order to be consistent with the ERK 1/2 experiments. An Aβ₁₋₄₂ treatment time-course study was also performed which showed there was a substantial increase in the phosphorylation of cPLA₂ at 15 minutes, 4 hours and 8 hours. (Figure 3.12)

Initially, a 15 minute Aβ₁₋₄₂ treatment time was used for cPLA₂ phosphorylation experiments, however there was no significant difference between the control group with no compound treatment and the Aβ₁₋₄₂ group with 15 minutes 5 μM Aβ₁₋₄₂ treatment. As a result, the experiment was repeated with 30 minutes 5 μM Aβ₁₋₄₂ treatment and an increase of approximately 1.5-fold of cPLA₂ phosphorylation was observed for bEnd.3 cells when compared to the control group (Figure
3.13, data contributed by both Andrey Tsoy and Tao Teng). bEnd.3 cells with ALP pretreatment prior to $\text{A}\beta_{1-42}$ treatment or ALP treatment alone showed no difference in p-cPLA2 production. Thus ALP is capable of suppressing $\text{A}\beta_{1-42}$ induced phosphorylation of cPLA2.
bEnd.3 cells were starved over a 24 hour period, p-cPLA2 level was measured periodically. The protein expression level was quantified by western blot analysis. The data is expressed as a fraction percentages of the control group with box plot from three independent experiments reported (* p<0.05, ** p<0.01, comparing with non-starving group).

**Figure 3.9 Starving time-course study on cPLA2 phosphorylation in bEnd.3 cells**
Figure 3.10 Aβ treatment time course study on cPLA₂ phosphorylation in bEnd.3 cells

bEnd.3 cells were treated with 5 μM Aβ₁₋₄₂ over a 22 hour period. p-cPLA₂ level was measured periodically. The protein expression level was quantified by western blot analysis. The data is expressed as true values from a single trial.
Figure 3.11 Azelnidipine suppressed Aβ-induced phosphorylation of cPLA₂ in bEnd.3 cells

bEnd.3 cells were treated with 20nM ALP for 4 hours, 5 μM Aβ₁-₄₂ for 30 minutes, both ALP and Aβ₁-₄₂ or neither compound (Control) to yield a total of four data sets. The protein expression level was quantified by western blot analysis. The data is expressed as a fraction percentage of the control group with mean ± SD from three independent experiments reported (**) p<0.01,
comparing with control group) (oo p<0.01, ooo p<0.001, comparing with 5 μM Aβ$_{1-42}$ treatment only group) (data contributed by both Andrey Tsoy and Tao Teng).
3.4 Azelnidipine Suppresses Aβ-induced translocation of NF-κB in bEnd.3 Cells

Translocation of NF-κB into the nucleus has been shown to induce the transcription of pro-inflammatory genes within endothelial cells as a result of Aβ<sub>1-42</sub> oligomerization. To test the effects of ALP on Aβ-induced NF-κB translocation, p65 subunit of NF-κB was labeled for immunofluorescence. Data showed that incubating bEnd.3 cells with 5 µM Aβ<sub>1-42</sub> for 2 hours led to an increase in translocation of NF-κB into cell nuclei. This effect was reduced by 35% when the cell cultures were pretreated with 20 nM ALP for 4 hours (Figures 3.3 and 3.4). A positive control group using TNF-α (instead of Aβ<sub>1-42</sub>) was employed to verify this technique. The results showed that treating with 100 ng/ml TNF-α led to a significant increase in NF-κB translocation into the cell nuclei, similar to that of the Aβ<sub>1-42</sub> treatment group (Figures 3.3 and 3.4). We also tested the effect of ALP alone, bEnd.3 cells pre-treated with 20 nM ALP for 4 hours showed no significant differences in NF-κB translocation when compared to the control group (Figures 3.3 and 3.4). Indeed, ALP suppresses the Aβ<sub>1-42</sub>-induced translocation of NF-κB within bEnd.3 cells.
Figure 3.12 Effect of ALP on Aβ-induced NF-κB translocation in bEnd.3 cells

bEnd.3 cells were treated with 20 nM ALP, 5 μM Aβ1-42, both ALP and Aβ1-42, neither compound (Control) or 100 ng/ml TNF-α (positive control) to yield a total of five data sets. The ratio of NF-κB translocation was quantified by dividing NF-κB p65 subunit intensity inside cell nucleus by intracellular NF-κB p65 subunit intensity. The data is expressed with the mean ± SD from four independent experiments reported (* p<0.05, ** p<0.01, comparing with control group) (o p<0.05, comparing with 5 μM Aβ1-42 treatment only group).
Figure 3.13 Representative immunofluorescent images of NF-κB p65 subunit and nucleus staining in bEnd.3 cells

Nucleus staining are represented in red color and NF-κB p65 subunit are represented in green color. The merged images were obtained by combining the nucleus staining and NF-κB p65 subunit of the same image. Compared to control (top images), Aβ increased translocation ratio of NF-κB (upper mid) which was suppressed by 20 nM Azelnidipine pretreatment (middle). 20 nM Azelnidipine pretreatment did not affect NF-κB translocation ratio (lower mid). TNF-α treatment group served as a positive control group (bottom).
Chapter 4 Discussion

ALP is a dihydropyridine compound with anti-inflammatory properties as a calcium channel blocker and has been used to treat patients with hypertension. Reported here is an extensive study of ALP as a potential treatment to alleviate the inflammatory and oxidative stress effects of Alzheimer’s disease. The data presented shows that ALP is capable of suppressing the production of ROS, the phosphorylation of ERK 1/2, cPLA$_2$ and the translocation of NF-κB within bEnd.3 endothelial cells that occurs as a result of Aβ$_{1-42}$ oligomerization. Taking the signaling/activation pathway into account we will investigate the mechanism by which ALP reduces the cytotoxic effects of Aβ$_{1-42}$. 
Figure 4.1 Schematic of relative pathways

Aβ<sub>1-42</sub> combines with RAGE which triggers NADPH p47<sub>phox</sub> subunit translocation and activates NADPH pathway. The ROS productions created through NADPH pathway further activates ERK 1/2 and cPLA<sub>2</sub> phosphorylation. ALP shows a competitive combination effect to RAGE with Aβ<sub>1-42</sub>, also suppresses activation of downstream pathway. Aβ<sub>1-42</sub> also initializes NF-κB translocation into cell nucleus which is attenuated by ALP. There also might be a cross-talk between ERK 1/2 and NF-κB.

Our data indicated that ROS production induced by Aβ was significantly suppressed by ALP in bEnd.3 cells (Figure 3.2). Also, we have data shows that ALP abrogates Aβ-induced co-localization between the cytosolic (p47-phox) and membrane (gp91-phox) subunits of...
NADPH oxidase (data not shown). We also present data for ALP suppression of Aβ-induced activation of ERK 1/2 (Figure 3.9 & Figure 3.10) and cPLA₂ phosphorylation in bEnd.3 cells, data also shows ALP significantly suppressed phosphorylation of cPLA₂ (Figure 3.13). All these evidences suggest that ALP may attenuate Aβ-induced co-localization of NADPH oxidase gp91-phox and p47-phox subunits and its downstream pathway, including ROS generation, ERK activation and cPLA₂ phosphorylation.

It is interesting that when we did time-course study on Aβ-induced phosphorylation of ERK 1/2 and cPLA₂, we found that cPLA₂ gave two responses at 15-30 minutes and 4-8 hours (Figure 3.12), but ERK 1/2 had only one response at ~15 minutes (Figure 3.7 & Figure 3.8). This likely means that cPLA₂ phosphorylation occurs via a different pathway at times greater than 30 min after ERK 1/2 activation. Previous reports have shown that oxidative stress up-regulates the NMDAR on bEnd.3 cells (Betzen et al., 2009). Also, NMDA evokes a Ca²⁺ increase in neurons which contributes to ROS production and cPLA₂ phosphorylation (Shelat et al., 2008). This may also happen in cerebral endothelial cells explaining the additional phosphorylation of cPLA₂ at 4-8 hours. Furthermore, Aβ has been shown to alter the concentration of intracellular Ca²⁺ in neurons and astrocytes (Sberna et al., 1997, Abramov et al., 2004, Niu et al., 2009, Rossner et al., 1997). Aβ may
have a similar effect on cerebral endothelial cells which would explain the ROS suppression properties of ALP shown here. Indeed, ALP may attenuate phosphorylation of cPLA$_2$ by blocking Ca$^{2+}$ channels.

Previous data suggests the receptor for advanced glycation endproducts (RAGE) plays an important role in Aβ-induced NADPH oxidase complex assembling, ROS generation, subsequent activation of ERK pathway and cPLA$_2$ phosphorylation in bEnd.3 cells (Askarova et al., 2011a). Our data also indicates that ALP competes with Aβ for binding to RAGE (not shown).

In bEnd.3 cells, NF-κB activation induced by cytokines has been reported (Menden et al., 2013, Pueyo et al., 2000). As we mentioned above, Aβ triggers activation of ERK 1/2 through ROS production induced by NADPH oxidase in bEnd.3 cells (Askarova et al., 2011b). Our data shows that Aβ also actives NF-κB translocation into cell nucleus in bEnd.3 cells and this is suppressed by ALP pretreatment (Figure 3.3 & Figure 3.4). So, we further postulated that there might be a cross-talk mechanism between ERK 1/2 and NF-κB pathways induced by Aβ in bEnd.3 cells. We know that the NF-κB translocation initiates from the activation of IκB kinase (IKK), which degrades IκB. Then NF-κB subunits are free to translocate to the cell nucleus and start gene transcription. Due to the above evidence, we suggest that ERK 1/2 is involved in Aβ-
induced NF-κB translocation, through ERK 1/2 or ROS cross-talk with IKK (Menden et al., 2013).
Chapter 5 Conclusion and Future work

This study shows that ALP is capable of suppressing the effect of Aβ-induced ROS production, ERK 1/2 activation, cPLA$_2$ phosphorylation and NF-κB translocation. This study proves that ALP is capable of alleviating two major factors that contribute to the cytotoxicity of amyloid-β in AD, oxidative stress and inflammation. This evidence suggests that ALP has the potential to be a pharmaceutical therapy for Alzheimer’s disease.

Future work should include further investigation of the potential cross-talk between ERK 1/2, ROS and NF-κB which can be accomplished using specific inhibitors for ERKs and ROS. Since cPLA$_2$ phosphorylation increased at both 15 minutes and 4-8 hours, it would be beneficial to test the effect of ALP on phosphorylation of cPLA$_2$ with 6 hours Aβ incubation in the presence of another calcium channel blocker as a negative control. Also, E-selectin is involved in NF-κB activation, so it would be interesting to design an experiment that would test the effects of ALP and Aβ$_{1-42}$ on both E-selectin expression and the cell inflammatory response.
References


53


H. Menden, E. Tate, N. Hogg and V. Sampath (2013) LPS-mediated endothelial activation in pulmonary endothelial cells: role of Nox2-


